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Original Article

Identification of clinical isolates of *Acinetobacter baumannii* from Iran and study of their heterogeneity

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Abstract

Background: *Acinetobacter baumannii* has become one of the most serious causative agents of nosocomial infections due to its significant ability to survive on hospital surfaces. It is mainly an emerging opportunistic pathogen infecting patients in intensive care units. This study was aimed to identify the clinical isolates of *A. baumannii* and to investigate their heterogeneity using polymerase chain reaction (PCR)-based typing methods.
Methods: A total of 197 nonduplicate isolates recovered from a wide range of clinical samples were subjected to conventional cultural and biochemical tests. For those isolates that were preliminary identified as *A. baumannii, rpoB*-based PCR with subsequent restriction fragment length polymorphism (RFLP) using two restriction enzymes (*Tag*I and *Hae*III) was performed to investigate the genetic diversity of the strains and their presumptive relationships with different clinical presentation of the disease caused by this pathogen.
Results: In total, 50 isolates (25.4%) were identified as *A. baumannii* using conventional phenotypic methods with subsequent confirmation by *rpoB* sequencing. RFLP analysis demonstrated five different restriction enzyme patterns, designated as A–E clusters. Most *A. baumannii* isolates were categorized under Cluster A (32%). We found no significant relationship between clinical presentation and the clustering of the isolates.
Conclusion: This study showed that the *rpoB* region possesses high discriminatory power to identify the isolates to the species level. This marker showed high interspecies variability that might be useful for strain typing. The results also suggest the possibility of the existence of a predominant clone of *A. baumannii* among infected patients in Iran.

Keywords: Acinetobacter baumannii; heterogeneity; nosocomial infections; *rpoB*

1. Introduction

Although acinetobacters are strictly aerobic Gram-negative coccobacilli that are widely distributed in soil and water, they are also commonly found in the hospital environment. Over the past 20 years, *Acinetobacter* species have emerged as opportunistic pathogens that are associated with severe hospital-acquired infections.[1,2] In particular, among the various species of this genus, *Acinetobacter baumannii* is responsible for a significant proportion of nosocomial infections.[3] The management of infections caused by *Acinetobacter baumannii* is greatly hindered by its intrinsic and acquired resistance to a wide variety of antimicrobial agents. In addition to this, the number of multidrug-resistant strains has increased during the past two decades.[4] Therefore, *A.*

Conflicts of interest: The authors declare that they have no conflicts of interest related to the subject matter or materials discussed in this article.

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baumannii has emerged as one of the most troublesome pathogens for health-care institutions globally. To control the spread of *A. baumannii* in the hospital setting, it is necessary to identify potential reservoirs of the organism and the modes of transmission. In addition, to distinguish *A. baumannii* strains involved in the outbreaks from epidemiologically unrelated strains, a comparison of isolates at the subspecies level is required by application of molecular typing methods.[2,5]

There are several typing methods for *Acinetobacter* spp., including ribotyping, polymerase chain reaction (PCR) hybridization with species-specific probes, pulse field gel electrophoresis, and random amplified polymorphic DNA typing.[6,7] The main disadvantage of these typing methods is low reproducibility, especially in terms of global molecular epidemiology. Using sequence-based typing such as single or multilocus or whole-genome sequencing provides more reliable data to compare strain information from a local point of view or globally. However, high cost is the main disadvantage of sequence-based typing in developing countries.[6,8] From this point of view, definitive identification of *A. baumannii* strains and investigation of their heterogeneity will be of value for both clinical studies and molecular epidemiology purposes. In this study, *rpoB*-based PCR sequencing was evaluated for definitive identification of *A. baumannii* strains and investigation of their heterogeneity. The presumptive relationship between genotypes and clinical presentation of the patients was also investigated.

2. Methods

2.1. Bacterial strains and phenotypic tests

A total of 197 nonduplicate isolates from a wide range of clinical samples from laboratories of the university teaching hospitals in Ahvaz and Tehran, Iran, were collected from November 2011 to January 2013. The preliminary proposal of the work was reviewed and approved by the Institutional Review and Ethics Board of the Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. We also received necessary permission for sample collection and starting the work. The sources of clinical samples and patients' medical histories are summarized in Table 1. The isolates were all those identified as *Acinetobacter* spp. based on the results of preliminary conventional phenotypic tests including growth on MacConkey agar, sugar fermentation, motility, catalase and oxidase tests, and other standard recommended tests.[9,10] For definitive identification of these isolates to the species level, molecular methods were used in the next step.

2.2. Molecular methods

The isolates were identified to the species level using species-specific *rpoB* gene-based PCR as previously described.[11] In brief, a 350-bp fragment of the *rpoB* gene was amplified from each isolate using two primers of 696F (5′-TAY CGY AAA GAY TTG AAA GAA G-3′) and 1093R (5′-CMA CAC CYT TGT TMC CRT CA-3′). To investigate the heterogeneity of *A. baumannii* isolates, restriction fragment length polymorphism (RFLP) was performed as described by other investigators, using *Tag*I and *Hae*III restriction endonucleases.[12]

2.3. Data analysis of rpoB gene sequences

The obtained sequences of the *rpoB* region of each strain were aligned with the published *rpoB* region sequences of *A. baumannii* strains retrieved from GenBank database using the JPhydit software package according to the primary structure.[13] Comparative analyses of the *rpoB* region were performed with the distance matrix, maximum parsimony, and maximum likelihood methods as implemented in the Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (MEGA4) program.[14] Tree topologies were tested by bootstrap analysis on 1000 replicates.

3. Results

A total of 50 isolates (25.4%) were identified as *A. baumannii* using conventional phenotypic methods with subsequent confirmation by *rpoB* sequencing. These 50 isolates were recovered from 20 male adults (40%), 14 female adults (28%), and 16 infants (32%). RFLP analysis demonstrated five different restriction enzyme patterns, designated as A–E clusters (designated as “isolate sequence type or seqtype”). These clusters had 98.2–100% similarity with the *A. baumannii* type/reference strain in GenBank (CIP70.34). A majority of *A. baumannii* isolates were categorized as Cluster A (32%; Table 2). Twenty-eight isolates (56%) were recovered from patients with pulmonary disease (Table 1). About half of the isolates that originated from pulmonary diseases were classified under Clusters A and B (53.57%), and all the isolates from meningitis cases, though the number was low, were classified under Clusters C and D. However, significant association between *A. baumannii* seqtype and age and sex of patients, pulmonary disease, and other clinical presentations was not seen.

A dendrogram based on maximum parsimony analysis reflecting the *rpoB* sequence-based clustering of all test strains of *A. baumannii* is shown in Fig. 1. Within the consensus tree, five clusters with distinct branches among the *A. baumannii* reference strains could be defined. The branches were supported with the highest bootstrap value (100%).

4. Discussion

In recent times, *A. baumannii* has emerged as a main opportunistic pathogen, and there is a high incidence of morbidity and mortality related to *A. baumannii* infection among immunocompromised hospitalized patients.[15] This organism is known for its involvement in hospital outbreaks and has sometimes caused interinstitutional spread.[5] Several studies have demonstrated the usefulness of *rpoB* gene sequencing for the identification and taxonomic classification of various bacterial species.[16,17] This study showed that the

Table 1
Characteristics of 50 clinical isolates of *Acinetobacter baumannii* from Ahvaz and Tehran, Iran.

Isolates	Sample source	G/A	PMH	Main symptoms	Section	*rpoB seqtype sequevars**
1	Endotracheal tube	M (42)	Chronic bronchitis	Fever, chest pain, cough	ICU	A
8	UC	M (44)	Kidney infection	FUO	ICU	C
11	Endotracheal tube	M (65)	COPD	Fever, urinary infection	ICU	E
12	Endotracheal tube	M (34)	Lung infection	Fever, cough	ICU	A
13	Ulcer	Neo	Subcutaneous ulcer	Dermatitis skin, purulent wound	NICU	A
16	Endotracheal tube	M (32)	Chronic bronchitis	Fever, cough	ICU	A
26	Endotracheal tube	F (28)	Lung infection	Fever, cough	General	A
27	UC	F (25)	Urinary tract infection	Fever, urinary infection, dysuria	ICU	A
29	Blood	Neo	Pneumonia	Fever, lung infection	NICU	E
31	CSF	F (29)	Brain abscess	FUO, headache, septicemia	ICU	A
35	Urine	M (35)	Kidney infection	FUO	ER	A
40	Endotracheal tube	M (38)	Lung infection	Fever, cough	General	A
41	UC	Neo	Bladder wall infection	Fever, urethritis	NICU	A
45	UC	Neo	Urinary tract infection	Fever, urinary infection	NICU	B
46	Blood	M (15)	Pneumonia	Fever, lung infection	ER	A
50	Blood	F (38)	Brain abscess	FUO, septicemia	ICU	B
51	UC	Neo	Kidney infection	FUO	NICU	C
53	UC	Neo	Urinary tract infection	Fever, urinary infection	NICU	A
54	UC	M (72)	Kidney infection	Fever, dysuria	General	A
55	Blood	Neo	Brain abscess	FUO, septicemia	NICU	E
58	UC	M (28)	Bladder wall infection	Fever, urethritis, dysuria	ICU	D
60	BAL	M (22)	COPD	Fever, chest pain, cough	ICU	E
61	Endotracheal tube	F (42)	COPD	Fever, cough	ICU	A
63	Endotracheal tube	M (20)	Pneumonia	Fever, cough	ICU	B
64	Endotracheal tube	Neo	Lung infection	Fever, cough	NICU	B
65	Endotracheal tube	F (68)	Pneumonia	Fever, chest pain, cough	ICU	D
66	Pleural effusion	M (72)	Pneumonia	Dyspnea, cough	ICU	B
69	BAL	Neo	COPD	Fever, cough	NICU	B
70	Endotracheal tube	Neo	Lung infection	Fever, cough, sputum	NICU	B
71	Ulcer	M (42)	DM	Swelling left leg, purulent wound discharge	ICU surgery	B
72	Ulcer	F (39)	Burning ulcer	Fever, soft tissue abscess	ICU surgery	E
73	BAL	M (42)	Chronic bronchitis	Fever, cough	ICU	B
74	Endotracheal tube	M (52)	COPD	Dyspnea, cough	ICU	B
76	Blood	M (19)	Meningitis	FUO, headache	Surgery	C
80	Blood	M (22)	Meningitis	Fever, weight loss, headache	ER	C
81	CSF	F (48)	Meningitis	Fever, headache, septicemia	Surgery	C
89	Ulcer	M (39)	Burning ulcer	Swelling left leg, purulent wound discharge Swelling left leg, purulent wound discharge	Surgery	C
93	Endotracheal tube	F (70)	Chronic bronchitis	Fever, cough	ICUB	C
95	BAL	M (56)	COPD	Fever, chest pain, cough	ICUB	E
104	Endotracheal tube	F (35)	Chronic bronchitis	Fever, chest pain, cough	ICUB	C
116	Endotracheal tube	Neo	Pneumonia	Fever, cough, dyspnea, cough	NICU	C
117	Sputum	Neo	Pneumonia	Fever, weight loss, headache	NICU	D
126	Exudates	Neo	Burning ulcer	Fever, septicemia	Neonatal	D
129	BAL	Neo	COPD	Cough, thoracic pain, weight loss	NICU	D
137	Endotracheal tube	Neo	pneumonia	Cough, thoracic pain, weight loss	NICU	D
150	Endotracheal tube	F (32)	Pneumonia	FUO	ICU	D
153	CSF	F (24)	Meningitis	Fever, chill, headache	ICU	D
161	BAL	Neo	Lung infection	Fever, chest pain, weight loss	NICU	A
313	Endotracheal tube	F (25)	Pneumonia	Fever, cough, chest pain	ICUB	A
470	Blood	F (25)	Brain abscess	FUO, septicemia	ICU	B

BAL = bronchoalveolar lavage; COPD = chronic obstructive pulmonary disease; CSF = cerebrospinal fluid; DM = diabetes mellitus; ER = emergency room; F = female; FUO = fever of unknown origin; G/A = gender/age; ICU = intensive care unit; M = male; Neo = neonatal; NICU = neonatal intensive care unit; PMH = past medical history; UC = urine culture.

Table 2
Restriction patterns and clustering of Iranian clinical isolates of *Acinetobacter baumannii* by *rpoB* RFLP and their similarity with type strain.

Isolates and designation	RFLP		Similarity with type strain %	RFLP type
	*Hae*III	*Taq*I		
1,[a] 26,[a] 40,[a] 41,[a] 161,[a] 313,[a] 12, 13, 16, 27, 31, 35, 46, 53, 54, 61	100/170/220/250/300/400	100/220/300/320/480	100	A
45,[a] 50,[a] 470,[a] 63, 64, 66, 69, 70, 71, 73, 74	100/170/220/250/300/400	220/300/320/450	99	B
8,[a] 51,[a] 76, 80, 81, 89, 93, 104, 116	100/170/220/250/300/400	220/300/320/460	99.7	C
58,[a] 65,[a] 117, 126, 129, 137, 150, 153	100/170/220/250/300/400	220/300/320/480/500	99.5	D
11,[a] 29,[a] 55,[a] 60,[a] 95,[a] 72	100/170/220/250/300/400	220/300/320/470	98.2	E

RFLP = restriction fragment length polymorphism.
[a] Representative of clinical isolates of *Acinetobacter baumannii* to drawing tree.

rpoB region has high discriminatory power to identify the clinical isolates of this pathogen to the species level and is of value for heterogeneity analysis. Our findings also suggest that a large variety of *A. baumannii* seqtypes may be responsible for causing diseases in humans, of which certain seqtypes seem to be more predominant. In our study, we identified and constructed phylogenetic trees incorporating 50 genospecies of *A. baumannii* based on the partial *rpoB* sequences following the RFLP analysis. RFLP demonstrated five different restriction enzyme patterns, designated as A–E clusters (seqtypes), and showed homogeneous grouping of *A. baumannii*.

From the total tested isolates that were initially identified as *Acinetobacter* spp. by phenotypic tests, only a small number was actually confirmed to be *A. baumannii* by a subsequent molecular examination. Although, in general, phenotypic tests are not reliable enough for the identification of *Acinetobacter* spp. isolates to the species level,[18] these tests exceptionally offer high sensitivity in identification of *A. baumannii* strains. This study showed that it is possible to achieve 100% confirmation of the isolates recovered (25.4%) as belonging to *A. baumannii* by application of confirmatory *rpoB*-based PCR followed by subsequent sequencing. The *Acinetobacter* spp.

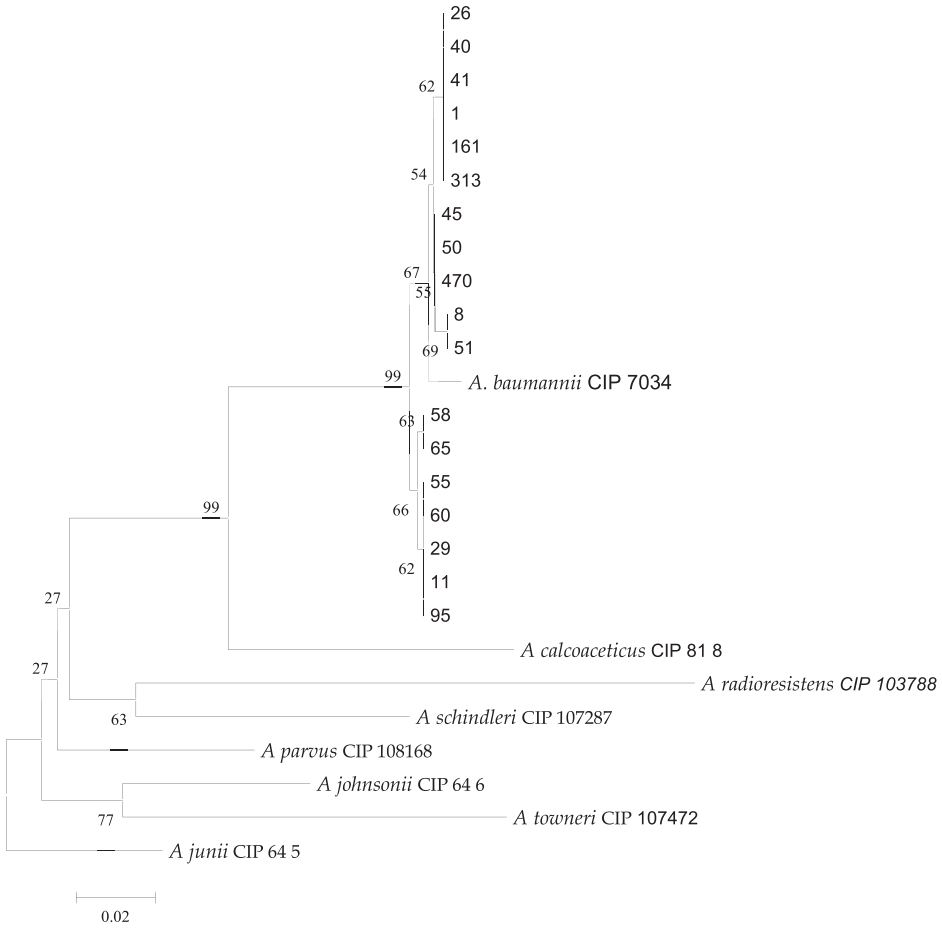

Fig. 1. Distance matrix tree showing the divergence of *rpoB* sequences of the clinical isolates of *Acinetobacter baumannii*. All alignment positions that are occupied by residues were used for the calculation of binary distance values. The topology of the tree was evaluated and corrected according to the results of maximum parsimony and maximum-likelihood analyses. The bar represents 0.1 estimated sequence divergence.

isolation rate in Iran varies from one setting to another. One significant report, conducted by Rahbar et al[19] from the Reference Health Laboratory of Iran, demonstrated the isolation of 88 isolates of *A. baumannii* in a 1-year survey in a reference hospital in Tehran. In another similar study, Rahbar and Hajia[20] reported the isolation rate of *Acinetobacter* spp. from nosocomial infections to be 3−6%. Moreover, in other similar studies, 78% and 8.8% of isolates recovered were, respectively, confirmed as *A. baumannii* by application of molecular techniques,[21,22] but these rates were not similar to that of our study. The difference in the obtained rates among these studies may be explained by the type of clinical sample, the methods adopted for sample processing and DNA extraction, and other factors that affect the rate of positivity.

In this work, the *A. baumannii* strains were categorized into five different clusters by application of the RFLP technique. Our study result was concordant with that of Karah et al,[22] who also reported five clusters for the *A. baumannii* isolates recovered on the basis of *rpoB* PCR and RFLP analysis. However, in the study by Gundi et al,[16] among the 32 *rpoB* seqtypes identified for the *Acinetobacter* spp., *A. baumannii* strains were grouped into 14 distinct clusters, which was more discriminative than our study or the clustering presented by Karah et al.[22] By contrast, in the study by Turton et al,[21] only three clusters were generated, showing less heterogeneity among their *A. baumannii* isolates. The confirmation of *A. baumannii* identification and clustering in our study and other mentioned works were achieved by comparing similarities of up to 97−100% with the *A. baumannii* reference strain. However, this similarity was reported to be wider, 77−100%, in the study by Turton et al.[21]

The species variation among *A. baumannii* strains revealed by RFLP enabled us to investigate the correlation between different seqtypes with demographic information and clinical presentation of the patients. Although the majority of patients who entered the study had some pulmonary complications and, from ~56% of these patients, strains belonging to *A. baumannii* seqtypes A and B were isolated, we could not find a significant correlation between the *A. baumannii* seqtype and clinical complication or age and sex of the patients.

In conclusion, this study showed that the *rpoB* region possesses high discriminatory power to identify the isolates to the species level. This marker also showed high interspecies variability when combined with the RFLP technique, which might be useful for strain typing. The results also suggest the possibility of the existence of predominant clones of *A. baumannii* affecting patients in Iran. Although some results in this study did not show statistical significance, it still provides groundwork for future studies.

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References

1. Sieniawski K, Kaczka K, Rucińska M, Gagis L, Pomorski L. *Acinetobacter baumannii* nosocomial infections. *Pol Przegl Chir* 2013;**85**:483−90.
2. Peleg AY, Seifert H, Paterson DL. *Acinetobacter baumannii*: emergence of a successful pathogen. *Clin Microbiol Rev* 2008;**21**:538−82.
3. Gaynes R, Edwards JR. Overview of nosocomial infections caused by gram-negative bacilli. *Clin Infect Dis* 2005;**41**:848−54.
4. Poirel L, Nordmann P. Carbapenem resistance in *Acinetobacter baumannii*: mechanisms and epidemiology. *Clin Microbiol Infect* 2006;**12**:826−36.
5. Griffith ME, Ceremuga JM, Ellis MW, Guymon CH, Hospenthal DR, Murray CK. *Acinetobacter* skin colonization of U.S. Army soldiers. *Infect Control Hosp Epidemiol* 2006;**27**:659−61.
6. Bartual SG, Seifert H, Hippler C, Luzon MA, Wisplinghoff H, Rodriguez-Valera F. Development of a multilocus sequence typing scheme for characterization of clinical isolates of *Acinetobacter baumannii*. *J Clin Microbiol* 2005;**43**:4382−90.
7. Ahmed SS, Alp E. Genotyping methods for monitoring the epidemic evolution of *A. baumannii* strains. *J Infect Dev Ctries* 2015;**9**:347−54.
8. Rafei R, Pailhoriès H, Hamze M, Eveillard M, Mallat H, Dabboussi F, et al. Molecular epidemiology of *Acinetobacter baumannii* in different hospitals in Tripoli, Lebanon using bla OXA-51-like sequence-based typing. *BMC Microbiol* 2015;**15**:103.
9. Forbes BA, Sahm DF, Weissfeld AS. *Bailey & Scott's diagnostic microbiology.* 12th ed. St Louis, MO: Mosby; 2007.
10. Golanbar GD, Lam CK, Chu YM, Cueva C, Tan SW, Silva I, et al. Phenotypic and molecular characterization of *Acinetobacter* clinical isolates obtained from inmates of California correctional facilities. *J Clin Microbiol* 2011;**49**:2121−31.
11. La Scola B, Gundi VA, Khamis A, Raoult D. Sequencing of the *rpoB* gene and flanking spacers for molecular identification of *Acinetobacter* species. *J Clin Microbiol* 2006;**44**:827−32.
12. Gomila M, Gasco J, Busquets A, Gil J, Bernabeu R, Buades JM, et al. Identification of culturable bacteria present in haemodialysis water and fluid. *FEMS Microbiol Ecol* 2005;**52**:10−4.
13. Zhang W, Sun Z. Random local neighbor joining: a new method for reconstructing phylogenetic trees. *Mol Phylogenetics Evol* 2008;**47**:117−28.
14. Tamura K, Dudley J, Nei M, Kumar S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* 2007;**24**:1596−9.
15. Howard A, O'Donoghue M, Feeney A, Sleator RD. *Acinetobacter baumannii*: an emerging opportunistic pathogen. *Virulence* 2012;**3**:243−50.
16. Gundi VA, Dijkshoorn L, Burignat S, Raoult D, La Scola B. Validation of partial *rpoB* gene sequence analysis for the identification of clinically important and emerging *Acinetobacter* species. *Microbiology* 2009;**155**:2333−41.
17. Zhan Y, Yan Y, Zhang W, Chen M, Lu W, Ping S, et al. Comparative analysis of the complete genome of an *Acinetobacter calcoaceticus* strain adapted to a phenol-polluted environment. *Res Microbiol* 2012;**163**:36−43.
18. Wang J, Ruan Z, Feng Y, Fu Y, Jiang Y, Wang H, et al. Species distribution of clinical *Acinetobacter* isolates revealed by different identification techniques. *PLoS One* 2014;**9**:e104882.
19. Rahbar M, Mehrgan H, Haji-Aliakbari N. Prevalence of antibiotic-resistant *A. baumannii* in a 100-bed tertiary care hospital in Tehran, Iran. *Indian J Pathol Microbiol* 2010;**53**:290−3.
20. Rahbar M, Hajia M. Detection and quantitation of the etiologic agents of ventilator-associated pneumonia in endotracheal tube aspirates from patients in Iran. *Infect Control Hosp Epidemiol* 2006;**27**:884−5.
21. Turton JF, Shah J, Ozongwu C, Pike R. Incidence of *Acinetobacter* species other than *A. baumannii* among clinical isolates of *Acinetobacter*: evidence for emerging species. *J Clin Microbiol* 2010;**48**:1445−9.
22. Karah N, Haldorsen B, Hegstad K, Simonsen GS, Sundsfjord A, Samuelsen O. Species identification and molecular characterization of *Acinetobacter* spp. blood culture isolates from Norway. *J Antimicrob Chemother* 2011;**66**:738−44.